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CRISPR-Cas9 technology to correct genetic disorders in embryos.

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ABSTRACT

The discovery of the CRISPR/Cas system has provided scientist with an efficient, easy and site-specific method for gene editing. The main constraint in genetic modification was to create a double-stranded break in the DNA and to replace the mutated gene with the minimum possible off-target effects.

Here, we systematically review the origin, function and uses of CRISPR-Cas9 technology for gene editing. The literature reviewed shows its uses are endless, from agricultural modifications to biotechnological applications.

We also demonstrate CRISPR-Cas9's potential with a case study *in silico* that replaces a mutated exon in the BRCA1 gene. This mutated exon is considered a pathogenic variant for hereditary breast and ovarian cancer and the idea is to correct it and prevent its transmission to the patient's children. After identifying the genetic alteration that needs to be repaired, with the help of the corresponding online tools, we have designed highly specic guideRNAs (gRNA) and a corresponding donor. The selected gRNA, complementary to the target DNA sequence, guides the Cas9 protein to the desired location with a high on-target efficacy and reduced off-target effects.

This technology has raised many ethical concerns that should be taken into consideration and are being examined worldwide such as the possibilities for germline editing and its ecological impact.

Keywords:

- Genetic modification
- CRISPR-Cas9
- Genetic engineering
- Treatments in embryos

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INTRODUCTION:

Genetic engineering or genetic modification is the manipulation of the DNA in any organism. This could be achieved in different manners including altering one base pair (A-T or C-G), inserting a copy of a gene, deleting a region of DNA or extracting DNA from one microorganism to combine it with another organism's DNA.

For decades, genetic modification studies were limited to the analysis of spontaneous mutations. Then, Muller (1927) and Auerbach (1947) proved that with the use of radiation or chemicals the rate of mutagenesis increased (1,2). Later methods relied on the use of viral vectors to transfer genetic materials into the cells. Once introduced into the nucleus of the host cell, the new genetic material was incorporated randomly into the genome. Therefore, these methods of genetic modification were unreliable as the insertion site could not be controlled (3).

The first genetic modifications with a specific target were achieved in yeast and mice (4,5) as a result of the process of homologous recombination. This is the exchange of nucleotide sequences between molecules of DNA with similar base sequences. This technique was precise but not efficient and was mainly based on the use of stem cells.

The main constraint in genetic modification was the ability to create a double-stranded break in the DNA, at the desired location and to replace the mutated gene with a minimum number of off-targets.

During the 1990's, it was found that specific nucleases could create a double-stranded break in the DNA on a specific site in the genome (6). The first enzymes used were the zinc finger nucleases (ZFN), which have a cleavage and a DNA binding domain, and were proved useful for site-specific modifications of the genome in fruit flies and mice (6,7). By 2005, this technology was used to correct a mutation in a human cell (8). However, programming ZFNs to target a specific site was a slow process and not fully reliable. Several ZFNs did not efficiently recognize the target DNA sequences while others cut DNA sequences that were similar to its target, resulting in non desired mutations or even cell death.

By 2009, scientists started using transcription activator-like effector nucleases (TALENs) from the proteobacteria Xanthomonas for genetic modification (9,10). They were remarkably effective compared to the ZFNs but as ZFN they required a different protein for each target DNA site and it was still a lengthy process.

All these studies showed a great potential in gene therapy research and for future treatments to many important human diseases. However, the processes were still time-consuming and costly. Thus, it was not until 2012 that the CRISPR-Cas system applications were discovered and an effective site-specific method of gene therapy was possible.

What is CRISPR Cas?

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) are DNA sequences joined with Cas (CRISPR-associated) proteins. This acronym pertains to the unique palindromic DNA sequences that are part of bacteria's and other microorganism's genome. These sequences are generally depicted as diamonds and squares. The diamonds represent the short repeats whilst the squares represent the spacers that interrupt the repeats regularly. Although CRISPR seemed innocuous, it was later discovered to be an essential piece of a bacteria's immune system against viruses.

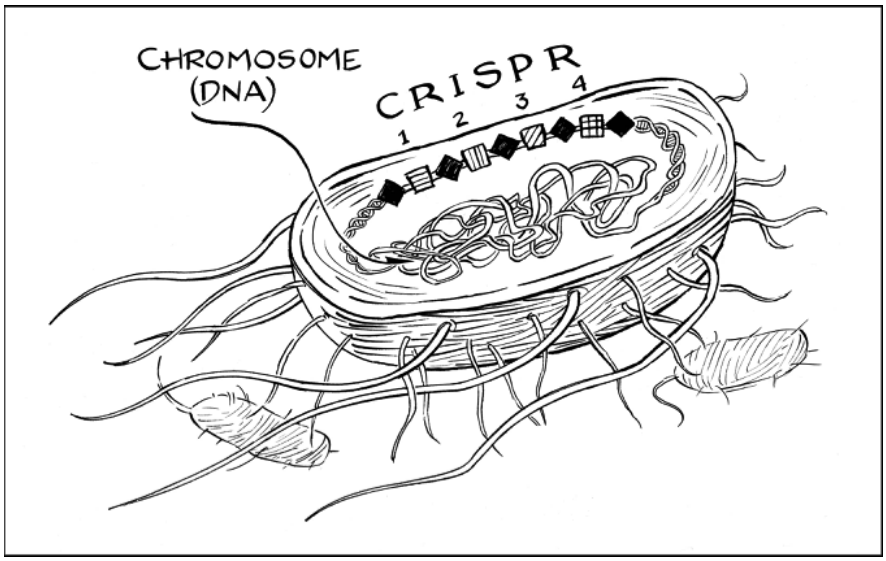


Fig. 1: CRISPR inside a bacterial cell. From (127)

The CRISPR-Cas system has the ability to recognize previous viral infections that the cell has come into contact with and uses the Cas protein to split the virus's DNA and destroy it (see Fig. 2). The virus's genome is essential for its replication, therefore, by splitting the virus's DNA, the CRISPR system ensures bacteria will not endure an ongoing viral infection.

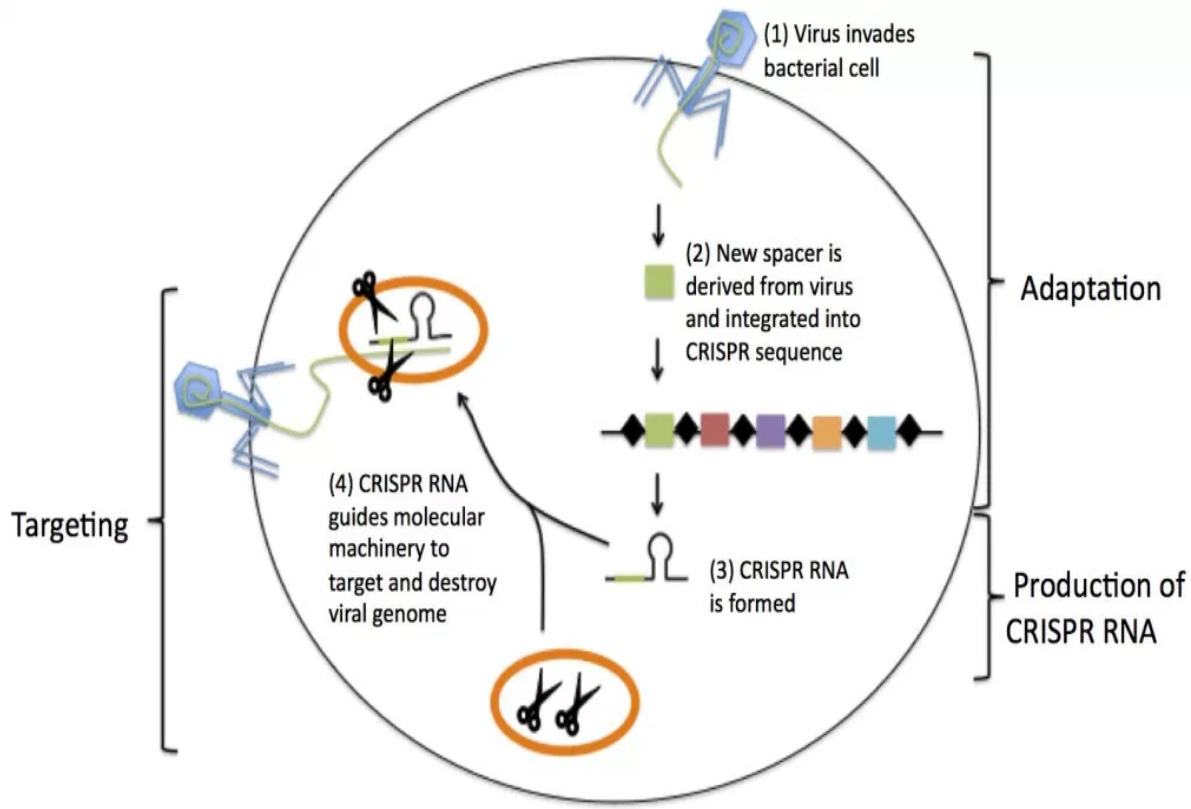


Fig. 2: *Simple steps that show CRISPR's immunity. From (134).*

This system would then be studied in depth so it could be used as an effective tool for genome editing.

OBJECTIVES:

1- To understand the origin, function and possible uses of the CRISPR/Cas technology for genome editing.

2- To demonstrate the potential of the CRISPR/Cas technology with a case study *in silico* by replacing an exon with a single nucleotide polymorphism (SNP) in the BRCA1 gene responsible for the tumorogenic effect.

METHODOLOGY:

A systematic review, with inclusion and rejection criteria, was used to analyze the CRISPR's origin and function.

Inclusion criteria:

- Scientific articles with full text.
- Articles published in scientific magazines.
- Randomized clinical trials.
- Bibliographic reviews.
- Documents had to be available in English.
- Keywords:

  - CRISPR-Cas9 AND genetic modification.

  - CRISPR-Cas9 AND genetic engineering.

  - CRISPR-Cas9 AND treatments in embryos.

Rejection criteria:

- Articles that could not be obtained in full text.
- Documents that do not pursue the objectives intended.

The search was executed between the months of January 2018 and March 2018. It focused on papers published by some of the most prominent experts on the subject like Jennifer A. Doudna and Francisco J.M. Mojica.

The web search was accomplished by combining the different keywords in the PubMed database. PubMed is a free database with publications of biomedical and life sciences literature at the U.S. National Institutes of Health's National Library of Medicine. It includes more than 28 million citations from MEDLINE for biomedical literature, life science journals and online books. Citations may have links to its full-text content from PubMed Central.

A preselection of articles was executed through a reading of their abstracts, excluding those studies that didn't follow the inclusion criteria previously mentioned. Then, an exhaustive reading of the full text of the remaining publications was completed so they could be thoroughly analyzed and included in the systematic review.

The case study of BRCA1 gene:

Materials:

1. Crispr design available at http://crispr.mit.edu.
2. Human genome database available at http://genome.ucsc.edu.
3. The genomic sequence of the locus to be targeted.
4. SNP database available at https://www.snpedia.com.
5. Integrated DNA technologies webpage: https://eu.idtdna.com/pages/home.
6. Computer with an Internet connection.

Identifying your SNP in BRCA1:

It is imperative to identify the SNP that is causing an alteration on the BRCA1 gene. In this case study, the SNP database that was used was SNPedia: https://www.snpedia.com.

Using this database you learn the SNP's position, the effect this variation of DNA has on humans and a description of the genetic alteration that needs to be repaired.

Identification of target sites using CRISPR design:

As soon as you know the position of the SNP that is causing your genetic alteration you need to find it in a human genome database. The database used for this case study was: http://genome.ucsc.edu.

- Go to "genomes"
- Click on "Human CRCh37/hg19". So the page takes you to the "UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly"
- Here you can enter the position of your SNP.
- You then press "zoom out" until you can see the region that needs to be replaced. If the SNP is within an exon, it is wiser to replace the whole exon to repair this genetic alteration.
- Select 240-250 base pairs in each intron on either side of the exon to be replaced. Click "view" and then "DNA" so you can obtain the DNA sequences at each side of the exon that will be used for the homologous recombination event.

This will identify the CRISPR/Cas9 target sites as well as the donor DNA to replace the mutated exon.

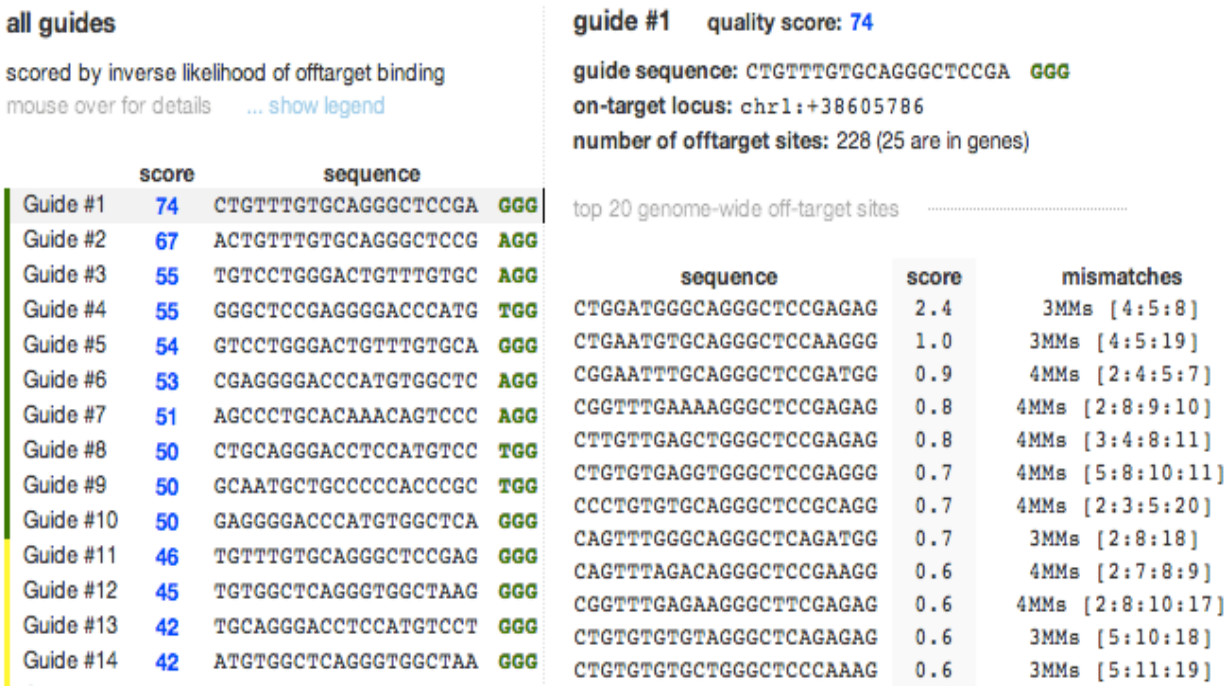
The “CRISPR design” is a web page where you can enter any DNA sequences and it assists you in the process of CRISPR guide selection. It shows any possible off-targets in the whole genome, highlights guides with high target specificity and orders all possible guides scored by the inverse likelihood of off-target binding.

To use the CRISPR design:

The database used for this study was: http:// crispr.mit.edu

- Go to the web page and enter your DNA sequence
- Choose a target genome. In this case study: human (hg 19)
- Click submit.

The DNA sequence will be processed to find possible CRISPR guides, 20 nucleotides adjacent to a PAM sequence: NGG. It will also find any possible off-target matches through the genome. Once this is all scanned you click “Job output” and you will obtain a list with all your possible guides scored by the inverse likelihood of off-target binding as well as a list of off- targets from each possible guide.



all guides

scored by inverse likelihood of offtarget binding

mouse over for details ... show legend

	score	sequence	
Guide #1	74	CTGTTTGTGCAGGGCTCCGA	GGG
Guide #2	67	ACTGTTTGTGCAGGGCTCCG	AGG
Guide #3	55	TGTCCTGGGACTGTTTGTGC	AGG
Guide #4	55	GGGCTCCGAGGGGACCCATG	TGG
Guide #5	54	GTCCTGGGACTGTTTGTGCA	GGG
Guide #6	53	CGAGGGGACCCATGTGGCTC	AGG
Guide #7	51	AGCCCTGCACAAACAGTCCC	AGG
Guide #8	50	CTGCAGGGACCTCCATGTCC	TGG
Guide #9	50	GCAATGCTGCCCCCACCCGC	TGG
Guide #10	50	GAGGGGACCCATGTGGCTCA	GGG
Guide #11	46	TGTTTGTGCAGGGCTCCGAG	GGG
Guide #12	45	TGTGGCTCAGGGTGGCTAAG	GGG
Guide #13	42	TGCAGGGACCTCCATGTCCT	GGG
Guide #14	42	ATGTGGCTCAGGGTGGCTAA	GGG

guide #1 quality score: 74

guide sequence: CTGTTTGTGCAGGGCTCCGA GGG

on-target locus: chr1:+38605786

number of offtarget sites: 228 (25 are in genes)

top 20 genome-wide off-target sites

sequence	score	mismatches
CTGGATGGGCAGGGCTCCGAGAG	2.4	3MMs [4:5:8]
CTGAATGTGCAGGGCTCCAAGGG	1.0	3MMs [4:5:19]
CGGAATTTGCAGGGCTCCGATGG	0.9	4MMs [2:4:5:7]
CGGTTTGAAAAGGGCTCCGAGAG	0.8	4MMs [2:8:9:10]
CTTGTTGAGCTGGGCTCCGAGAG	0.8	4MMs [3:4:8:11]
CTGTGTGAGGTGGGCTCCGAGGG	0.7	4MMs [5:8:10:11]
CCCTGTGTGCAGGGCTCCGCAGG	0.7	4MMs [2:3:5:20]
CAGTTTGGGCAGGGCTCAGATGG	0.7	3MMs [2:8:18]
CAGTTTAGACAGGGCTCCGAAGG	0.6	4MMs [2:7:8:9]
CGGTTTGAGAAGGGCTTCGAGAG	0.6	4MMs [2:8:10:17]
CTGTGTGTGTAGGGCTCAGAGAG	0.6	3MMs [5:10:18]
CTGTGTGTGCTGGGCTCCCAAAG	0.6	3MMs [5:11:19]

Fig. 3: *Job output page from “CRISPR design”.*

When a guide is chosen, the top 20 genome-wide off-target sites, are displayed. Off-targets are organized by their affinity to the selected guide, taking into account the number of mismatches and the position of each mismatch, which are then highlighted in the DNA sequences.

Guides with a score of 50 or higher are highlighted in green, this means they are considered good options as a targeted sequence. You then have to check that there aren't any gene regions with a high off-target score. Guides highlighted in yellow could be used as backups if there are no suitable green guides, but they are not ideal. If, like in this case, you are trying to make a cut on the introns around an exon, you could look further up or down the DNA sequence, to see if there are any guides highlighted in green. Guides highlighted in red should be avoided because they have many off-target interactions.

Designing of the Donor:

Once you have your guides, you need a donor sequence. The donor sequence will be the one to replace the DNA sequence with the mutation. For this to happen, the donor needs to have the whole exon, without the mutation, and homology arms of 200 base pairs.

- Go to the web page: http://genome.ucsc.edu.
- Select all the DNA between one guide and the other.
- Click "view" and then "DNA".
- Add 200 base pairs on each side
- Copy this DNA sequence.
- Change your PAM sequences, so the Cas9 nucleases will not cut your donor sequence once it integrates.

To obtain your donor you need to submit your DNA sequence to a company for synthesis. IDT (Integrated DNA technologies) is amongst the most specialized companies in this regard and the one preferred by many laboratories. They will then deliver your sequence as a single-stranded DNA.

- Go to the web page: https://eu.idtdna.com.
- Click on "products and services" and "single-stranded DNA fragments" to obtain your donor.
- Click on "CRISPR genome editing" and "Alt-R CRISPR-Cas9 System" to obtain your RNA-guides.

"Alt-R CRISPR-Cas9" provides essential tools for genome editing studies. You can also obtain Cas9, tracrRNA and RNA guides by providing them with your guide sequences.

Once you've bought all the required materials from "Alt-R CRISPR-Cas9" you could carry out your experiment in vitro. Zygotes with this precise mutation could be edited by inserting this genome editing tools through electroporation.

Systematic review:

Origin

The history of CRISPR-Cas began in 1987 in Japan, when Atsuo Nakata's group found a previously unidentified structure in *Escherichia coli* (Gram-negative bacteria) that consisted of palindromic structures separated by similarly sized spacers (11,12).

A similar structure was discovered in the *Mycobacterium tuberculosis* complex (Gram-positive bacteria) (14). These are bacteria with a big genetic distance from *E.Coli.* However, the importance of this connection was not realized until 1989, when Francisco Mojica, from the University of Alicante (Spain), found in archaea: *Haloferax Mediterranei* similar structures to those reported by Nakata's group. Mojica found a structure with short palindromic repeats of 30 bp separated by spacers of around 36 bp (13) and realized that, if such similar complexes were present in microbes with such a vast evolutionary distance, they must be of great importance.

From 1996 to 1999, Mojica discovered many more of these structures in other archaea and bacteria. By 2000, he had found them in 20 different microorganisms and named them Short Regularly Spaced Repeats (SRSR) (14). The study of SRSR's loci in many archaea and bacteria led to the discovery of four genes close to the SRSR, cas1- cas4, that were presumed to have a related function (15).

In 2002, Mojica changed the acronym to CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) after the bioinformatic analyses established that the palindromic repeats occurred in clusters, with spacers of the same extent lengthwise (15, 16).

CRISPR is a part of the microorganism's immune system

Mojica then focused on the spacers between the repeats. After introducing them into the BLAST program, he found a match between one spacer and a bacteriophage that infected *E. coli*. Therefore, he was the first to suggest that CRISPR were homologous to the viruses that infected those bacteria. He revealed, that those bacteria with fragments of viruses inserted in their genomes, were resistant to the infection of those particular viruses (17). Other laboratories confirmed this theory(18,19) and they all concluded that CRISPR must be part of bacteria's immune systems (17).

The sequences extracted from viruses and stored by the bacteria between the spacers were referred to as protospacers. Studying the spacer sequences they realized there was a small part of DNA retained next to the protospacers, which would later be referred to as PAM (protospacer adjacent motif) (20). They hadn't yet realized why PAM was important but it was certainly essential for the CRISPR system, since it appeared next to all the protospacers.

Parallel to this study, Phillippe Horvath, a Ph.D. student at the University of Strasbourg, started working for Rhodia Foods. Horvath wanted to eliminate the frequent bacteria infections that affected the dairy fermentation process. Therefore, he focused his studies in cresting a method to detect precise strains of bacterial DNA resistant to viral attack. Once he learned about CRISPR, he started working with Rodolphe Barrangou and Sylvain Moineau to test the hypothesis that the CRISPR was part of the bacteria´s immune system (21).

In 2007, they were able to confirm their hypothesis with a study using the bacteria *Streptococcus thermophilus* and two bacteriophages. They conducted a series of experiments where they added the spliced phage's DNA into the bacteria's CRISPR region. They then checked if these bacteria were resistant to that particular bacteriophage (21). Understanding how bacteria could recognize and destroy those phages during future infections was the key to acquiring immunity against the bacteriophages in the dairy industry.

This posed further enquiries such as: How was the genetic material being destroyed? How was it being recognized and targeted? All these questions led to the search of other components of the CRISPR system.

Focusing their studies on the proteins encoded by the *cas* genes, they discovered the Cas7 and Cas9 proteins and their functions. On one hand, Cas7 was required for the acquisition of new spacers but it was not involved in the cutting of the invading DNA. On the other hand, Cas9, previously referred to as csn1, produced a large protein with two nuclease motifs (HNH and RuvC). It was presumed to be the protein responsible for cutting the invading DNA (19, 22) and therefore essential for the bacteria's immunity.

While some scholars focused their studies on Cas proteins others looked into how the genetic material was being recognized and targeted. This led to many investigations about the RNAs possible implication in this system.

RNA's are a part of the CRISPR system

Stan Brouns, postdoctoral at the University of Wageningen in the Netherlands, and John van der Oost focused their studies on RNAs involvement in the CRISPR system. By 2008, they proved that CRISPR arrays were converted into long RNA strands, which matched the sequence of CRISPR's DNA. These long strands were then cut by an enzyme into shorter RNA strands of the same length. This smaller pieces of RNA (13,23, 24) differed from each other by the sequence contained in their spacers. Each spacer contained a sequence acquired from a different phage. Therefore, CRISPR RNAs (crRNAs) were essential for the bacteria's immunity (25). To prove the hypothesis that crRNA's made a complex with Cas proteins and were then guided to the viruses DNA, they started a study with *E. Coli* (25). In their study they programmed CRISPR (the first artificial CRISPR) to target four genes in the *E. coli* bacteriophage. Their results suggested that the *E. Coli* DNA was the target of this crRNA Cas complex, since it was then proved to be resistant to the virus.

Luciano Maraffini and Erik Sontheimer, also confirmed that DNA was the target of this CRISPR system. They were the first to realize this system could be remodeled and used for genome editing in eukaryotic cells (26).

Other discoveries about this system's function were made by many other experts in the field. Sylvain Moineau in collaboration with Danisco discovered Cas9 made a double-stranded cut in DNA. They also realized the PAM sequences were an important part of the process (27), since they appeared to be a recurring occurrence in many complexes (20,28). They noticed the DNA was always cut in the same location in relation to the PAM sequence.

Emmanuelle Charpentier and Jörg Vogel discovered that there was another small RNA implicated in the process. This RNA molecule was named trans-activating crRNA (tracrRNA) and it merged with crRNA. Once both RNAs merged the RNaseIII processed them into their mature products. Several experiments confirmed tracrRNA was crucial for the crRNA to function therefore essential for the CRISPR system (29). Later investigations showed tracrRNA had another function. It was essential for a bridge to be created between crRNA and the Cas proteins, so the Cas proteins could then cleave DNA (30,31).

Classifying CRISPR

The acquisition of progressively more information on the CRISPR Cas systems led to their classification. They were classified depending on their function and composition into type I, type II and type III (32,33).

Studies showed many Cas proteins in type I and type III systems (25, 34- 38), while type II systems only used one Cas protein (30, 39). This attribute that would later on prove really useful. Furthermore, the protospacer adjacent motif (PAM) was only a part of type I and type II systems (17, 20, 27, 40).

Since more proteins were discovered and their behavior was further analyzed, they created a new classification, where Class 1 was constituted of types I, III and IV while Class 2 included types II and V (41). Class 2 only needs one protein, Cas9, for it to recognize its target and make the necessary DNA cuts, while Class 1 need a multitude of protein complexes (42). TracrRNAs are also exclusive of Class 2 systems. Both these qualities explain why they selected type II CRISPR Cas systems to be studied in depth and tried to use its components to develop the technology necessary for targeted DNA cleavage.

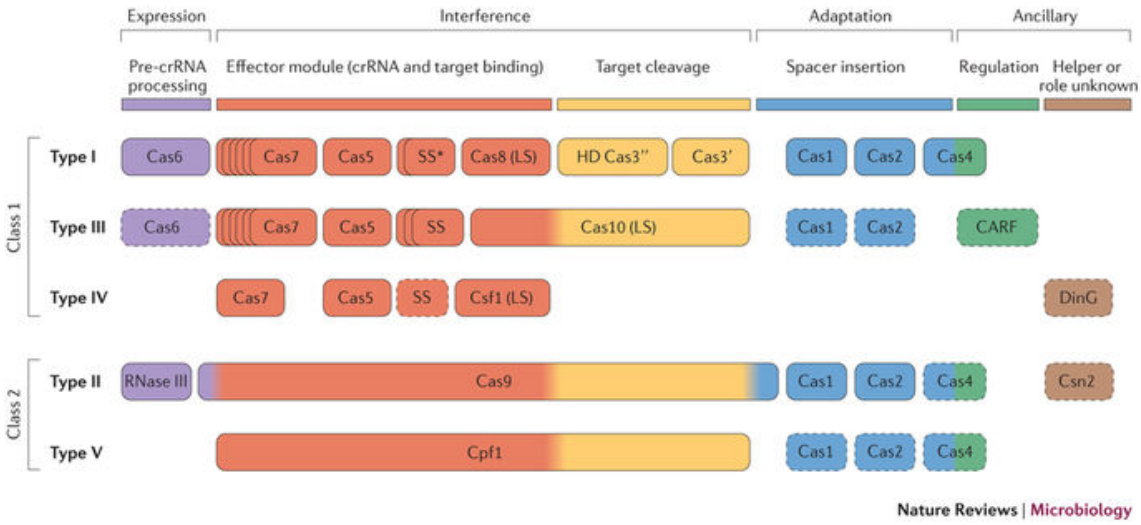


***Fig. 4**: Functional classification of Cas proteins. From (41).*

Transferring CRISPR between organisms

Vigjinijus Siksnys and collaborators, trying to examine if we had all the elements necessary for the CRISPR Cas system, conducted an experiment were the CRISPR Cas system from *S. thermophilus* was transferred to a microbe with a great genetic distance, *E. coli*. Until this study in 2011, the CRISPR Cas system had only been investigated in the bacteria that contained them. Their results were outstanding, the immunity got transferred from *S. thermophilus* to *E. Coli* (43). This confirmed that CRISPR Cas systems could be transferred between species, since these two microbes have a greater genetic distance than the one between humans and yeast (44, 45) and they could be adapted to target specific sequences (43).

This was a major breakthrough in the CRISPR study. Once all the constituents of the CRISPR system were recognized (crRNA, tracrRNA, and Cas9 proteins), scholars were ready to perform accurate biochemical investigations.

Crispr in vitro

By 2012 two teams of scientists assessed the behavior of CRISPR/Cas systems in vitro and both came up with the hypothesis that their components could be used to edit genomes (30,39).

Jennifer Doudna at the University of California Berkeley and Emmanuelle Charpentier in Sweden started working together and studied the function of *S. pyogenes* CRISPR-Cas9 system's components (Cas9, tracrRNA, and crRNA). They focused their study on how these molecules acted together to destroy viral DNA.

Isolating the Cas9 protein they demonstrated that it was able to cleave DNA in vitro. Cas9 proteins contained two domains (HNH and RuvC) which cut the opposite strands of the DNA, achieving a double-stranded break (DSB).

This cleavage took place in the specific DNA sequence homologous to the crRNA spacer region.

Furthermore, they employed effective custom-built crRNAs and verified both crRNA and tracrRNA were necessary for cas9 to accomplish its purpose. In addition, they established how tracrRNA and crRNA could be fused into a single guide RNA (sgRNA) retaining both of their properties. This extraordinary discovery made it possible for us to have an uncomplicated programmable RNA-guided genome editing tool (30).

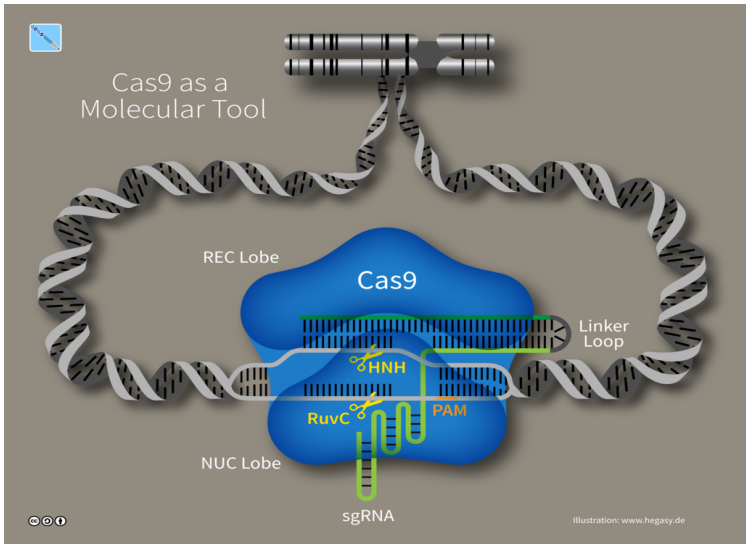


Fig. 5: *Cas 9 creates a double-stranded break guided by a single guide RNA.* From (135).

Around the same time, Siksnys, Barrangou, and Horvath worked together and studied the activity of *S. thermophilus'* type II systems in vitro (39). Like Doudna and Charpentier they proved that the DNA could be cut in vitro with a double-stranded cleave, three nucleotides away from the PAM sequence. In their research, they created custom-built spacers that would lead the Cas9 protein to cut whatever gene they chose. Their evidence also proved that the Cas9's NHN domain was responsible for the cutting in the DNA strand that was complementary to the crRNA, while the RuvC domain was responsible for the cut in the opposite strand. Moreover, they showed that the crRNA could be shortened up to 20 nucleotides and still be effective (31). They recognized the potential of this technology and suggested that it could be used to perform DNA surgery using programmable RNA guides (39).

Both teams published their findings by 2012, inspiring many scientists to prove that RNA- programmable DNA endonucleases could be used as a tool to edit the genome in mammalian cells (46).

Genome editing

Three main groups of investigators, one led by Luciano Marraffini (47), another by Feng Zhang (48), and the third one by George Church (49) focused their studies on the use of CRISPR technology to edit the mammalian genome.

Feng Zhang was working with TALENs when he learnt about CRISPR. He started using CRISPR Cas9 tools derived from *S. thermophilus* to use in human cells. By 2011, he used this technique to target the luciferase gene in human embryonic kidney cells, but the decrease in luminescence was small. So, he tried to improve the technique by considering ways of increasing the amount of Cas9 that accessed the nucleus. He became aware that the cas9 from *S. thermophilus* had an irregular dissemination within the nucleus and by testing alternatives he realized that the Cas9 from *S pyogenes* was more evenly distributed. He also discovered that even though human cells don't possess RNaseIII they could still use crRNA. By 2012, he had effective components for this technic: a CRISPR array, Cas9 from *S. pyogenes* and tracrRNA. In his investigations he targeted 16 loci in human and mice cells and found he could mutate genes, deleting genes by non-homologous end joining and inserting new genes by assisted recombination. He also realized several genes could be edited at the same time and when he read Charpentier and Doudna's investigation he tried the sgRNA that they used in their in vitro study. The sgRNA didn't work as expected in vivo, it didn't cut many of the loci, but he realized that a full-length fusion was effective (48, 50). Zhang showed how versatile this technology could be: it could be used to create personalized mice models of someone's specific genetic disease or cancer in weeks. It could also help us locate all the required genes for any biological system. Furthermore, he found more class 2 CRISPR systems, even one with a different nuclease than Cas9, that didn't need tracrRNA to function (51).

At the same time, George Church, a Harvard professor, who had collaborated with Zhang also did an investigation on how to edit the human genome. Like Zhang, he tested fusions of crRNA- tracrRNA and realized short fusions did not work out in vivo, while full-length fusions were effective. In his study, he targeted seven loci, mutating genes by non-homologous end joining and homologous recombination (49).

Using Church's sgRNA, Keith Joung, a Harvard professor, experimented with CRISPR technology on zebrafish. He determined it could be used to effectively produce mutations in the germline (52).

This were the initial studies, but later on, CRISPR Cas technology was used on several types of cells and organisms (Table 1).

BIOLOGY		BIOTECHNOLOGY		BIOMEDICINE
CELL LINES	MODEL ORGANISMS	CROP PLANTS	FUNGI	organoids
HEK293	Mice	Rice	Kluyveromyces	hESCs
U2OS	Rats	Wheat	Chlamydomonas	iPSCs
K562	Fruit flies	Sorghum		
	Nematodes	Tobacco		
	Arabidopsis			
	Salamanders			
	Frogs			
	Monkeys			

Table 1: First cell types and organisms that had their genomes edited with CRISPR-Cas technology.

This table continues to grow. Scientists have used CRISPR to modify the genome in hundreds of plants and animals.

It's hard to compare the effectiveness of the studies since they have got different target sites but there are analysis reporting that the efficiency can reach up to 80% or more, more than what we obtained using ZFNs or TALENs (52,53).

Table 2. Timeline of the key discoveries that led to the CRISPR-Cas9 technology (1987–2013)

Year	Discovery	Reference
1987	First discovery of CRISPR in Gram negative bacteria.	11
1991	First discovery of CRISPR in Gram positive bacteria.	14
1993	First discovery of CRISPR in archaea.	13
2000	CRISPR are named short regularly spaced repeats (SRSR)	14
2000	Many short regularly spaced repeats are discovered in several bacteria and archaea, which suggests an important pupose.	14
2002	Regularly spaced repeats acquire a different name CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)	16
2002	Discovery of Cas genes.	15
2005	First finding that CRISPR spacers matched sequences in bacteriophages and plasmids.	17
2005	First hypothesis that CRISPR-Cas systems were a part of the bacteria's immunity.	21
2007	Experiments prove that CRISPR-Cas systems are part of an adaptive immunity against bacteriophages.	21
2008	Experiments prove CRISPR Cas systems target DNA.	22
2008	First discovery that small RNA's form part of the CRISPR system as guides.	25
2008	They realize the protospacer adjacent motif (PAM) has a crucial role in the CRISPR-Cas system.	27
2010	First explanation about how DNA is cleaved at precise locations due to Cas proteins in the CRISPR-Cas system.	30
2011	Discovery of a new small RNA that was part of the CRISPR system, the trans-activating CRISPR RNAs (tracrRNAs).	29
2011	Transferring CRISPR Cas sytems to a genetic distant organism: from *S. thermophilus* to *E. coli.*	43
2012	First experiments to asses CRISPR Cas systems in vitro and scientist suggest it's components could be programmed and be used to edit the genome in mammalian cells.	30,39
2013	First successful experiments were CRISPR Cas's constituents were used to edit the genome of mammalian cells.	47,48,49

Mechanism of the CRISPR-Cas system:

The CRISPR systems in bacteria

Currently, the Type II CRISPR systems are the basis for the genome editing technology.

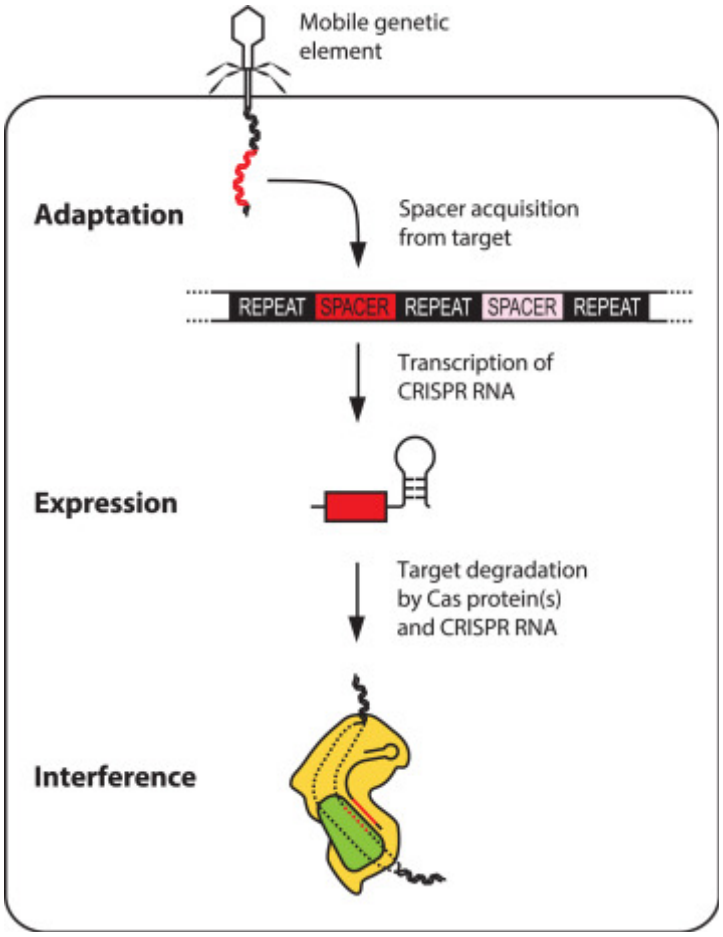


Fig. 6: *The key steps of CRISPR-Cas's immunity.* From (136)

The CRISPR Cas immunity process can be split into three phases: adaptation, expression, and interference (Fig. 6). The CRISPR contains short repeated sequences that are separated by spacers. These spacers are acquired from viruses DNA and this is what happens in the first step of the CRISPR Cas immunity process, adaptation. By attaching more spacers new viruses can be identified, therefore, more viruses genomes can be recognized and destroyed by the bacteria. Spacer acquisition modifies the genome, therefore the offspring will inherit the immunity.

Adjoining the CRISPR array there is a group of CRISPR associated genes that code for proteins indispensable in this immune system. Moreover, CRISPR is a chronological record of the viruses the bacteria have come into contact, due to the new spacers getting added at one side of the CRISPR. The key components of spacer integration are *Cas1* and *Cas2* genes and their subsequent proteins (21,25). This genetic memory is crucial for the following phases, expression, and interference, to destroy the re-invading genetic material from viruses.

During the second phase, expression, C*as* genes are expressed and their proteins formed. The CRISPR is also transcribed and a long precursor CRISPR RNA (pre-crRNA) is formed. The pre-crRNA is then processed by Cas proteins into small mature crRNAs. This crRNAs contain a single spacer each and the Cas protein responsible varies with each type of system (54).
Type 2 systems have a different mechanism than the other systems, for the biogenesis of crRNA. They transcribe a second RNA, tracrRNA, that pairs with the pre-crRNA and both get processed together by the RnaseIII (29) In addition, they have got another distinct feature, they require the Cas9 protein for its immunity (29, 30).
The comparison of the three types of CRISPR systems shows that Types I and III had different mechanisms to obtain their immunity, they used Cas6 proteins instead of Cas9 and processed pre-crRNA differently (34).

In the third stage, interference, the nucleic acid from the virus is recognized and eliminated with the aid of the crRNA and the Cas proteins. Target interference is based on the ability of the crRNA, bound to the Cas protein, to locate the corresponding spacer to set off the disintegration of the target. The disintegration is executed by specific Cas nucleases (25,55).
Types I and II systems need a PAM sequence present adjacent to the spacer and require a perfect complementarity between the spacer and the crRNA (56,57,58). The crRNA joins one of the strands of DNA by base pairing and the non bound DNA strand is displaced and forms an R-loop (59). The Cas protein then cuts the target DNA and degrades it. In Type I systems it is the Cas3 nuclease the one needed for interference (22, 25, 33), while type II systems only require Cas9 proteins. The Cas9 protein contains 2 nucleases domains the HNH and RuvC which will cleave the complementary and non-complementary strands of DNA, respectively (60).
Type II systems will also require the tracrRNA joined with the crRNA and Cas9 protein to perform the recognition and degradation of the target (29).
Type III systems undergo a different process to the PAM recognition in types I and II. Since these systems do not have PAM sequences, they recognize the target by extending the crRNA base pairing with the host DNA (61).

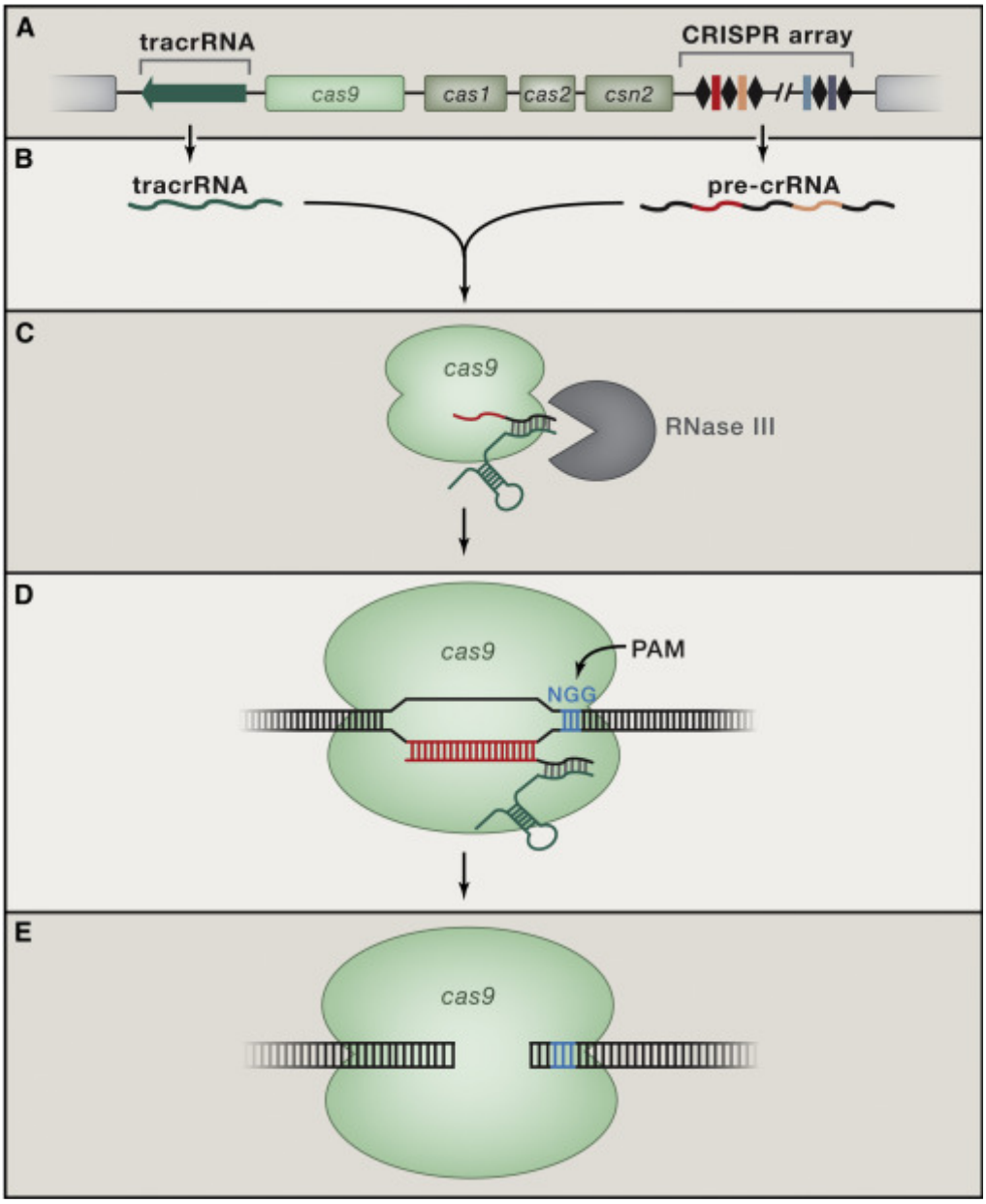


Fig. 7: *Class 2, Type II CRISPR- Cas9 System from Streptococcus thermophilus.* From (137).

(A) The locus consists of the CRISPR array with black diamonds representing the repeat regions and colored squares for the spacers, four genes (cas9, cas1, cas2 and csn2) that will code for four different proteins and the tracrRNA. (B) The CRISPR array and tracrRNA are transcribed so a pre-crRNA and a tracrRNA are formed. (C) Both RNAs merge and are cut into shorter pieces by Cas9 and RNase III. (D) There is a complex formed by Cas9, tracrRNA, and crRNA that will search for the matching DNA sequence from the spacer (showed in red). For this complex to bind to their target it requires a three nucleotide sequence named PAM, were the Cas9 will attach itself. (E) Once Cas9 it attached it will create a double-strand break as a result of its two endonuclease domains (HNH and RuvC) cutting opposite strands.

Applications of CRISPR technology for genomic editing

With this remarkable technology scientists found a simple way to rewrite any genome, and showed that the possibilities were endless. As long as the guide RNA matched the 20 bps in the DNA and the cas9 was present any cells' genome could be edited. Scientists used this new technology in many different ways:

- To functionally assess the mutations in mice genomes. Since mice are genetically speaking very related to humans and are small, they are the perfect test subjects for animal research. This is crucial to gain a better understanding of human diseases and test the efficacy of potential drugs or medical interventions (62, 63, 64, 65).

- To create genome edited primates (66).

- To improve the quality and quantity of crops, fruits, and vegetables. CRISPR has already been used to make wheat resistant to a certain type of bacteria (68), to confer potatoes the ability to not sweeten during storage (69), to generate soy plants that produce oils with less LDL (70) and to produce mushrooms that do not spoil prematurely (71).

- To edit the genome in livestock for biomedical [90] and agricultural purposes [91]. Scientists have created cows with 20% more muscle, with less fat and more amount of meat (72). This small change in livestock's genomes can result in an increase in the production of food. Their genome can also be edited to make them healthier or have a better resistance to diseases. Other examples of gene-edited livestock are genetically dehorned cows (73), pigs with an increased resistance to viruses (74) and goats with longer hair (75).

- It is possible to inactivate multiple genes that could provoke a reaction from the human's immune system in porcine cells, for xenotransplantation purposes. There is a shortage of organs and many people die while they are waiting to receive a transplant. Even if this has not yet been done in any human clinical trials the results with baboon recipients have been promising (76).

- Thanks to CRISPRs ability to insert o delete part of a gene it can produce an alteration in the genes ability to create a functional protein. This is referred to as a gene knockout or KO. Therefore it is possible to manufacture a lentiviral knockout CRISPR library of thousands of single guided RNA sequences which are able to knock out specific genes. This facilitates the identification of genes required for a specific biological response as well as of potential drug targets for diseases (77,78,79).

- To build gene drives, so the DNA that is inserted into the cell contains the genetic information that encodes CRISPR. Scientists have already used the gene drives to give mosquitoes a resistance to *Plasmodium falciparum* and to spread genes for sterility in female mosquitoes, which could potentially eradicate the entire species. This technology could be used to eliminate many diseases which have mosquitoes as vectors, like malaria, dengue, Zika virus and many others (67). Recently, gene drives have also been reported in mice (131).

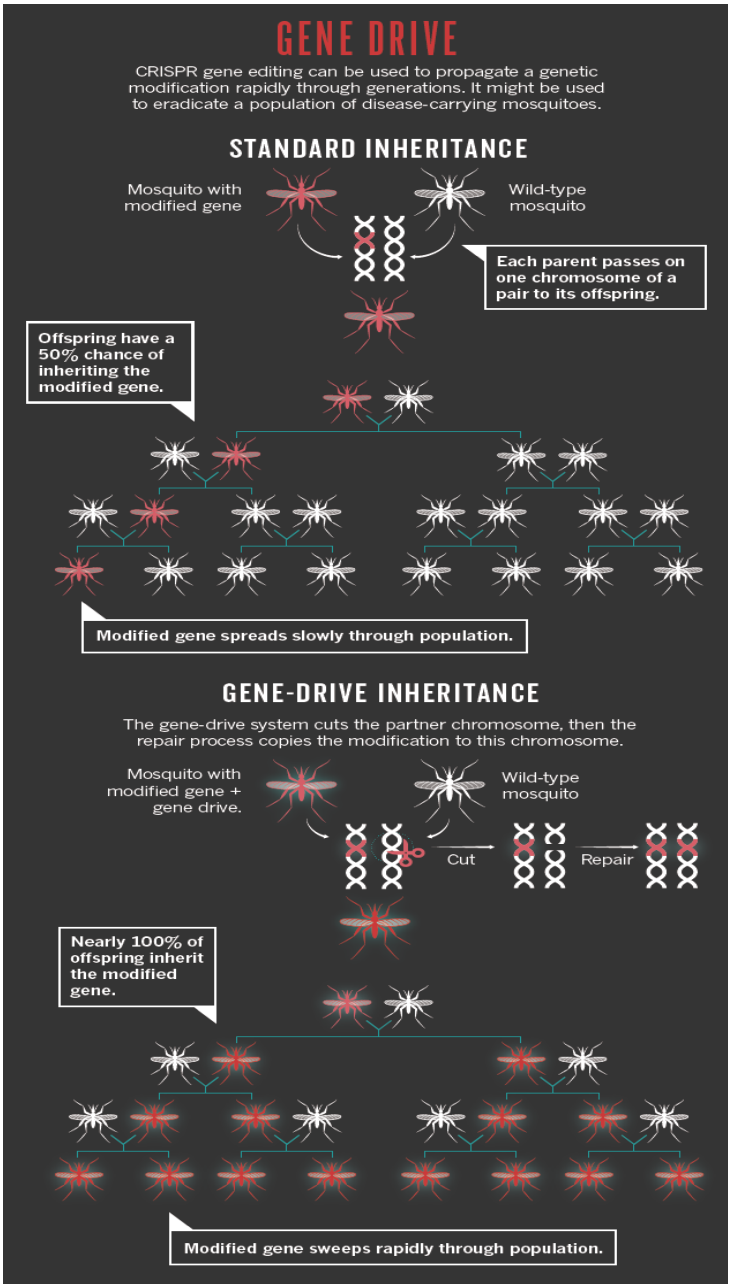


Fig. 8: *Comparing natural inheritance to gene drive inheritance.* From (138).

- To reproduce chromosomal rearrangements, large inversions, and translocations observed in patients that were extremely difficult to model in human cells (80) and in mice (81). This has made it possible for us to replicate a patient's cancer, such as lung cancer (82), acute myeloid leukemia (83) and Ewing's sarcoma (84). Thus, it's possible for us to study cancer's and other disease's development and progression.

- To edit nonviable human embryos so it is possible to study our genome (85).

- To create protocols for in vivo genome editing (86) and studying possible therapies for devastating genetic diseases in animal models and human cells. Some examples in which its use was successful are the modification of a mutation in the Crygc gene that was responsible for cataracts in mice (87) and to cure mice of other genetic affections such as muscular dystrophy (88, 89) and several metabolic disorders (90). In cultured human cells, CRISPR was able to repair many genetic diseases like sickle cell disease, hemophilia, cystic fibrosis, and severe combined immunodeficiency (91, 92, 93, 94) and very recently progeria (132).

- To edit the CCR5 gene and therefore preventing the HIV from latching onto it and to remove viral genes from infected cells (95).

- To further understand cancer and determine which mutations cause tumorigenesis quicker (96).

- To edit induced pluripotent cells (iPS) that can later be the ideal cells for autologous cell replacement on humans with a previous genetic disorder (97, 98).

- To create gene therapies for human diseases (99).

Other applications of CRISPR technlogy

- There are other applications to this technology, not just gene editing. Scientists created a version of Cas9 without its endonuclease activity, therefore without its ability to make a cut in the genome. It can still locate specific DNA sequences and once they are located gene expression can be controlled. This was called CRISPR interference (CRISPRi) and it can repress the expression of specific genes or even increase its output. It can prevent the RNA polymerase from binding, stop the transcriptional elongation or block a transcription factor. It has been used to repress the expression of multiple genes simultaneously, its effect can be reversed and it is highly specific (100,101,102).

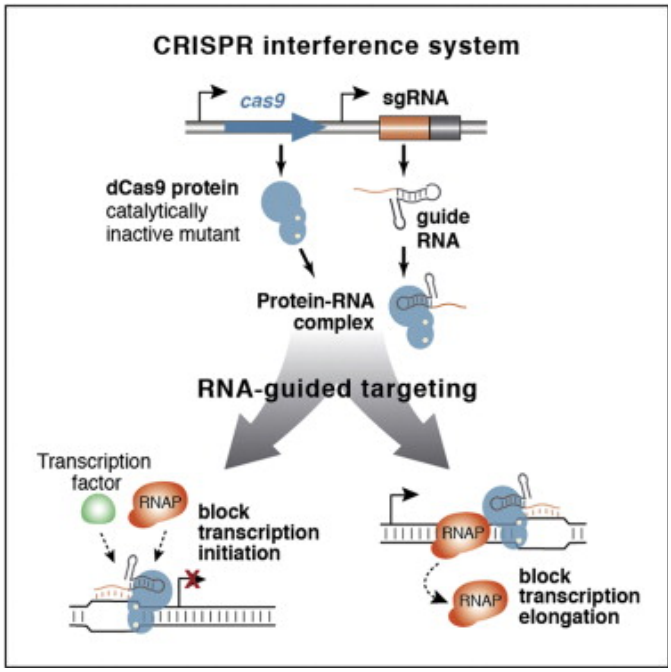


Fig. 9: *CRISPR interference system's use in controlling gene expression.* From (139).

- This inactive version of Cas9 can also be fused to a fluorescent protein so you can visualize genes involved in specific biological processes. (103)

CASE STUDY RESULTS:

Genetic alteration to be repaired:

This genetic alteration is a variant considered pathogenic for hereditary breast and ovarian cancer syndrome.

The SNPs database "SNPedia" describes its position, the exact alteration in the DNA and the recommended actions if it is clinically confirmed.

SNP: rs886040335

It is a single nucleotide variant (A; G). The mutated codon, TGA, produces a stop codon instead of the tryptophan that produces the non-mutated codon, TGG.

Nowadays the recommended action for someone with this mutation would be to:

- From 25 years onward, get intensive screening for breast and ovarian cancer.
- Bilateral mastectomy as a prophylactic surgery, to reduce the appearance and mortality from breast and ovarian cancer.
- First degree relatives should get tested for this mutation and should increase their screening.

BRCA1 variant considered pathogenic for breast cancer	
Is a	genotype
of	rs886040335
Gene	BRCA1
Chromosome	17
Position	43.094.568

Magnitude	6
Repute	Bad

Geno	Mag	Summary
(A; G)	6	BRCA1 variant considered pathogenic for breast cancer
(G; G)	0	common in clinvar

TABLE 3: Summary of the case study's SNP in the BRCA1 gene.

Design of the gRNAs:

With the exact position of this SNP (43.094.568), you can go to the genome database to locate it. Once you zoom out you see this SNP is a part of the exon 9 in the BRCA1 gene. This is the exon we have to try to replace by cutting the DNA on the introns at both its sides. To cleave at both sides, two guide RNAs need to be designed.
By using CRISPR design you can obtain all possible guides scored by the inverse likelihood of off-target binding. The best guides would be “guide 1” at both sides, both ideal candidates since they have a quality score above 50 and are highlighted in green. This will be the gRNAs needed to guide the Cas9 nuclease to the exact point in the genome where it needs to cut.

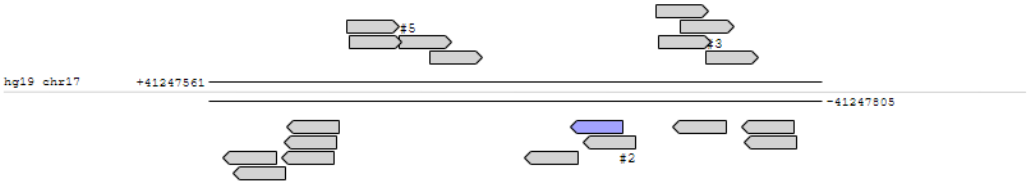


Fig. 10: *All possible guides at the left side of exon 9 from the BRCA1 gene. Guide one is showed in purple.*

Guide 1:

- quality score: 92
- guide sequence: CTCTGTCAAATGTCGTGGTA(TGG)
- On target locus: chr17:- 41247704
- number of off-target sites 91 (9 in genes)

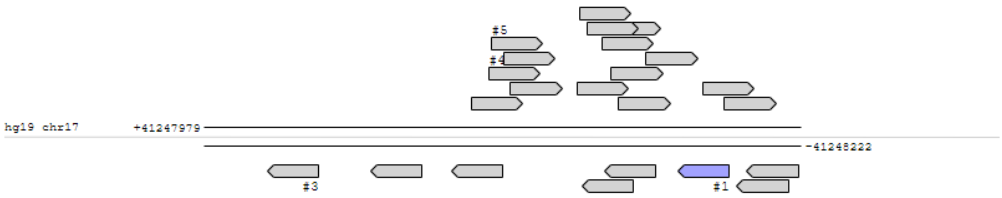


Fig. 11: *All possible guides at the right side of exon 9 from the BRCA1 gene. Guide one is showed in purple.*

Guide 1:

- quality score: 72
- guide sequence: CTTGAACTCATGACCTCAAG(TGG)
- On target locus: chr17: 41248171
- number of off-target sites 163 (10 in genes)

Design of the donor:

The DNA between both RNA guides needs to be replaced by a donor without the mutation. Using the genome database you can obtain the DNA sequence between both RNA guides and add 200 base pairs as homology arms.

ssDONOR
>hg19_dna range=chr17:41247504-41248381 5'pad=200 3'pad=200 strand=+ repeatMasking=none

TGGGAGGATAGCTTGAGCCTTGGAGGTGGAGGTTGCAGTGAGCCAAGATT
GCATCACTGCACTCCAGCCTGGGCAACAGAGTGAGACCCCATCTCAAAAA
AAAACAAACAAACAAACAAAAAAAAAAACGAAAGGGCAACAATCAGTTAC
AGAAGGTCTTATTATAGGTACATTAGTCTAGTACCATTTAAATCTATCAG
A**CCA**TACCACGACATTTGACAGAGAATGATACTCTAACTCTGCCAAGAGA
TTTTGTGGGTTGTAAAGGTCCCAAATGGTCTTCAGAATAATCTAATTACA
GTACTGTATCTACCCACTCTCTTTTCAGTGCCTGTTAAGTTGGCAAACTT
TGCCATTACCCTTTTTTGCAGAATCCAAACTGATTTCATCCC**TGG**TTCCT
TGAGGGGTGATTTGTAACAATTCTTGATCTCCCACACTATAGGGAAAAGA
CAGAGTCCTAATAAGAAACACTAGTTACATGTATGCAGAACTGTCAAATG
ACCAAGATCAAACATTTTAGCTCTTTCGATTACAGAAAGCTGACCAATCT
TATTTAGTTAGTGAAAGCTGCTCTCTCCTTTAGAAACTTCTAGTTGAAAT
GAAATGGTTGCTGGGCACGGTGGCTCAAGCCTGTAATTCCAGCACTTTGG
GAGGCTGAGGCGGGTGGA**CCA**CTTGAGGTCATGAGTTCAAGACCAGCCTG
GCCAACATGGTGAAACCCCATCTCTACTAAAAATACAAAAATTAGCCGGG
TGTGGTGGCGCGCGCCTGTAGTCCCAGCTACTAGAGAGGCTGAGGCAGGA
GTATGGCTTCAACCTGGGAGGCAGACGTTGCGGAGAGGTGAGATCACACC
TCTGTACTCCAGCCTGGGCAACAGAGCA

- Purple for the homology arms.
- Red for the RNA guides with the PAM sequence highlighted.
- Black for the DNA between both RNA guides.
- Green for the exon with its mutation highlighted.

This single stranded donor DNA is generally obtained from a company, in our case from IDT (Integrated DNA technologies). Before ordering it, both PAM sequences need to be changed, so Cas9 will not eliminate the donor once it is incorporated. Thus, the CCA changed to TGA. The rest of the donor sequence needs to stay exactly the same.

IDT can provide you with all the necessary tools to carry out this case study in vitro. You can buy Cas9 nuclease and get your exact gRNA and donor if you provide them with the precise DNA sequences.

Reagents are injected in the cytoplasm of the zygote, preventing the genetic alteration transmission to the patient's children. Alternatively, these can be injected in the pronucleus of the zygote, but cytoplasmic microinjection is simpler.

DISCUSSION:

Possibilities of the technology and perspectives:

Research on genome editing will continue until it becomes an invaluable tool in many aspects of our life. But will CRISPR-Cas9 be the definite programmable nucleases or will we find something more efficient?

CRISPR-Cas9 is a simple technology, with a single protein to cleave the DNA and recognition of the exact target by base pairing, so it is hard to envision a more efficient way to edit the genome. There is the possibility of finding a chemically-based reagent that combines the DNA split and the DNA recognition. This has been researched for decades, with many different proteins, without successful results (104,105,106). Therefore CRISPR-Cas9 and variations from this technology seem to be the future in genome editing.

There are many useful applications for this technology in the agricultural sector, with both livestock and crop plants. These genetically modified organisms differ from GMOs in crucial ways (107). Current GMOs are created by using different chemicals on seeds and selecting the plants with the desired traits, without being certain of other changes that might have occurred in the DNA of that plant. Now, due to this new technology we can select the desired genetic material and insert it in a precise location, making it a more accurate technique. Moreover, in most cases, these changes are ones that could have occurred naturally and there is no need to insert genetic material from other species.

The already edited crops like disease resistant wheat (68), mushrooms that do not spoil or soy plants that are more nutritious for human consumption (70) are just the starts to healthier and more proliferate crops. Cows edited to develop without horns (73) and sheep (72), pigs (108) and cows (72) with less fat and a higher percentage of muscle are the beginning of genome editing in livestock to improve animal and human welfare. The disruption of a single gene may result in an increase in food production. Will the public have a better acceptance of this technology than GMOs? Current aversion to GMOs is generally based on the greed and power of certain businesses, not on evidence of adverse effects. The public will have to decide what is more important, the process or the product? If CRISPR-Cas9 and its variations can protect livestock from deadly viruses, banish practices such as dehorning and increase food production we should consider the many benefits to both humans and animals before categorizing it as “another GMO”.

Beyond agricultural modifications, animals genome are being edited to produce models of human diseases to increase our knowledge of their biological processes and to test drugs and other therapies.(109) Companion species are likely to have their genome edited too, to create new breeds or to cure breeds of their genetic predispositions to certain ailments.(110)

Furthermore, a new line of work called “de-extinction” is being developed, with projects such as the Woolly Mammoth Revival in George Church’s laboratory. The idea that animals that have become extinct could be reintroduced into their natural habitat is an appealing concept to the scientific community. A genetic modification of an existing organism would have to take place, for example, to create the woolly mammoth several genetic changes are being introduced into the Asian elephant cells (111). Researchers will not only create the woolly mammoth, or some Asian elephant hybrid, they will do the same to many other species soon. The possibility of bringing back dinosaurs is still remote because nobody knows the DNA sequence that encodes them.

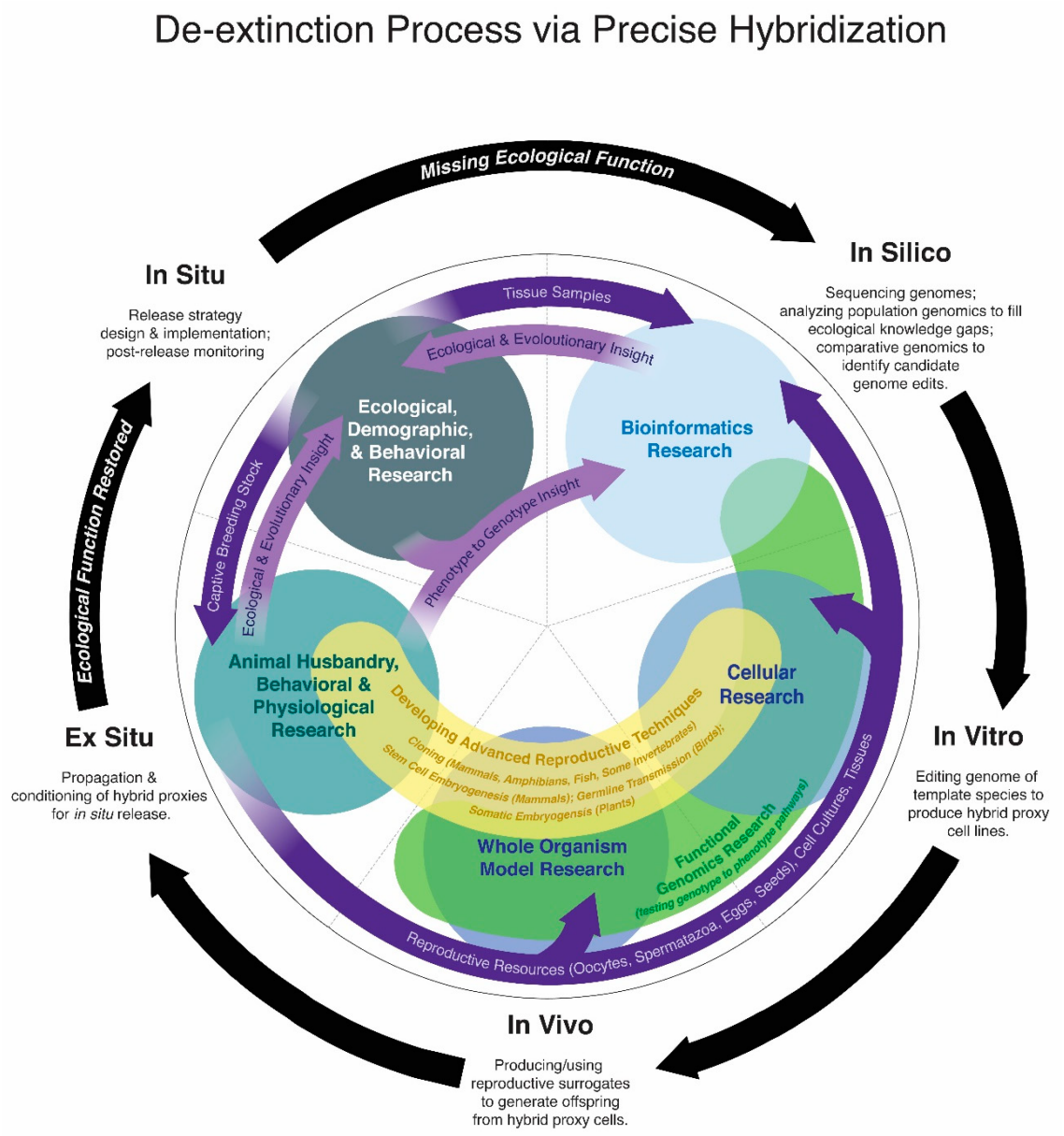


Fig. 12: *The de-extinction process starts in silico and ends in situ.* From (140).

Another future application for this technology relies on its use as gene drives. A genetic modification can spread through a population passing down the alteration to all its descendants. This is possible because the genetically modified animal contains the altered gene, the genes for the Cas9 enzyme and several guide RNAs that tell it where to cut. When the modified animal comes into contact with the wild species it modifies their DNA so all their descendants will contain the same alteration. This gene drives have already been developed in mosquitoes, producing sterility in their females (112) and inactivating genes required for parasites to grow (113). There are many countries with mosquito-borne diseases that could benefit enormously with this approach because it could reduce the transmission of malaria, dengue, Zika virus, etc. This has raised many concerns, even if these diseases could be eradicated, the consequences of eliminating an entire species are unpredictable (114, 115). We can not anticipate the consequences it would have on other species that rely on those mosquitoes. Moreover, we can not foresee if the drives will become ineffective by adaptation or mutation in the organisms. Laboratory tests can not predict the impact of gene drives, the only way to see reliable results is by releasing them into a natural environment. Therefore, in the near future humans will have in their hands the technology to easily eradicate whole species that are vectors to many human diseases. We will have to decide if the benefits outweigh the risks.

The possible medical applications for this technology are countless. CRISPR-Cas9 could be used in germline editing, so genetic diseases will not manifest on patients or their descendants, as well as in somatic therapies, to cure diseases in fully developed patients.

Somatic therapies greatest challenge is the delivery of the gene-editing technologies to certain cells in our organism, affected by a particular genetic disease. *Ex vivo* gene therapy is a simpler approach because cells can be removed from the patient, edited and then tested to see its efficacy before reintroducing them into the patient. Clinical trials to edit blood cells to cure beta-thalassemia and sickle cell disease are starting this year in Europe since there have been promising results with monkeys (116). Genome editing has also been used as a therapy to eradicate HIV. The first clinical trial used ZFNs to knock out the CCR5 gene, the one responsible for producing the CCR5 protein where HIV latches onto (117). Due to the encouraging results of this trial, other clinical trials were approved to remove HIV with CRISPR-Cas9 (118, 119). Although the experiments are just starting they are encouraging and this technology could prove to be the ultimate solution to cure HIV.

While *in vivo* gene editing is currently not ready for clinical trials because we have to figure out how to deliver CRISPR-Cas9 to the affected cells and not provoke an immune response, many scientists are working on a solution. Several means of transport are being considered, primarily the use of viruses as vectors or the use of nanotechnology. Adeno associated viruses (AAV) have been assembled to target cells in different tissues from the human body. With this vector, the CRISPR-Cas9 technology was able to repair muscle cells in mice with Duchenne muscular dystrophy (120) and to target the liver and cure mice of tyrosinemia (121). There are different AAV vectors that target lung, brain, and retinal cells, that could potentially be used in the near future to cure diseases such as Huntington, congenital blindness or cystic fibrosis, among many others.

Pharmaceuticals such as Bayer are trying to develop CRISPR based drugs to treat afflictions such as blood disorders, blindness, and heart disease. This could conceivably create a new line of treatments, an injection or pill that could possibly treat frequent genetic disorders (122).

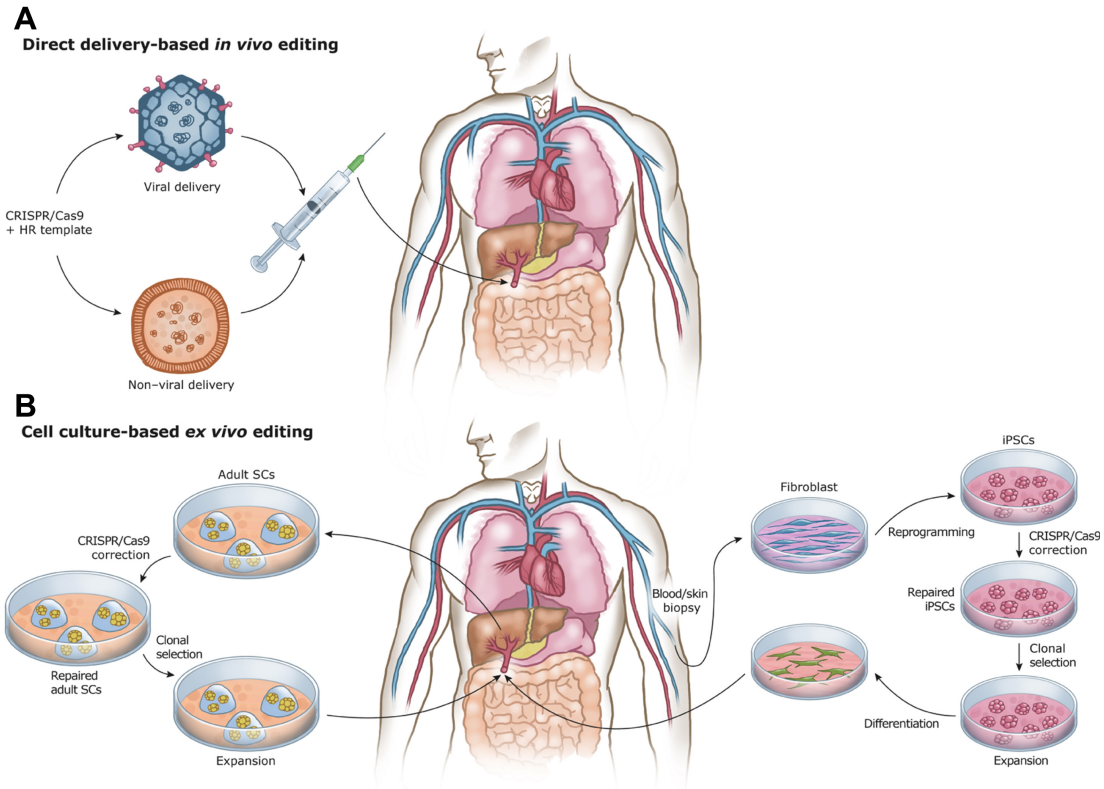


Fig. 13: *Different approaches for CRISPR-based gene therapies.* From (141) *(A) In in vivo editing, CRISPR-Cas9 components are injected directly into the patient using vectors for in situ gene editing.*
(B) In ex vivo, genes are edited in patient-derived cells and are transplanted back into the same patient after the correction.

Germline editing is also one of the most promising possibilities of this technology. The editing reagents can be introduced into the embryos created through in vitro fertilization (IVF). The biggest benefit of this process is it not only eliminates the disease from that individual but also from all its descendants. The problem with this technology is that, at present, it lacks specificity and efficiency to be completely safe. First, the results of genetically modified embryos needs a decrease in mutations generated at non-target sites before it can be applied to treat embryos, since any changes made with this technology will not be reversible (123). Despite this, there is already a case reported were two twin girls were born with edited genomes in China. He Jiankui was the first to alter the DNA of human embryos during in vitro fertilization (133). Since there are hundreds of scientists researching this line of work, it is easy to predict its use in the near future.

Once germline editing is a feasible treatment, many hereditary cancers, among other diseases, could be prevented. Some would be just as easy as a one letter change in the embryos genome, like the one shown in the case study analyzed in this paper.

Case study analysis:

CRISPR- Cas9 is a revolutionary technology thanks to its simplicity and specificity. In this case study, we outline the experimental work prior to the *in vitro* study of CRISPR-Cas9 in the BRCA1 gene. This pertaining SNP, rs886040335, is the one responsible for hereditary breast and ovarian cancer. A single nucleotide change, an A for a G, would repair the genetic alteration, resulting in a perfectly healthy patient.

For the *in vitro* study to be successful, reliable guide RNAs are essential, so they can guide the cas9 protein to the exact location that needs to be cut. In this case study the "CRISPR design" web page was used to help select both guide RNAs. It is a very reliable program which highlights guides with a high target specificity and arranges all possible guides by the inverse likelihood of off-target binding. Even so, the use of more than one web page to choose the optimal guide RNAs, would make the chosen guides more reliable and with fewer off-targets. Other internet programs to compare possible guide RNAs could be CHOP-CHOP or GenScript.

There is a significant amount of off-targets warranted by the Cas9 enzyme. Many scientists have created variants of the guide RNA or the Cas9 enzyme with an increased specificity (124). Church Zhang proved that the combination of two mutant Cas9 enzymes, each one cleaving a single strand of DNA, could be used to cut opposite strands, leading to a double-stranded cut with a reduced off-target activity (125). This modified Cas9 enzymes could be considered a viable alternative to improve this case study.

An important factor related to off-site targets is the amount of Cas9 enzyme. The appropriate amount of cas9 enzyme would have to be taken into consideration, before introducing all the reagents into the cell. A high concentration of Cas9 increases off-targets, whereas a low concentration diminishes the cleavage activity on the target (126).

Future studies will make progress in the accuracy of the technology by designing Cas9 variants, as well as further understanding the conformational rearrangements of Cas9 prior to target cleavage. This could all improve the site-specific insertion of new genetic information, making this case study even more reliable in vitro.

Ethical implications:

With the emergence of the CRISPR Cas system and all its potential, many ethical concerns arose. Some ethical issues to be considered were:

1- The circumstances in which CRISPR technology should be used and its safety.
2- The possibility of inheritable changes in the genome of human embryos.
3- If there should be an international organization or legislation created to regulate CRISPR applications.
4- The access to CRISPR technology.

While using this technology it is possible for off targets and mosaicism to occur, this makes safety one of the main concerns. Scientists and people who know of this technology, such as those present at the International Summit on Human Gene Editing in December 2015, concur that until it is a safe procedure it should not be used for clinical reproductive purposes. With the knowledge we have today, the risks of introducing unpredictable mutations in future generations are greater than its benefit as a treatment, therefore it would go against the principle of non- maleficence (127).

We must also consider that gene-editing also has secondary effects. Editing the CCR5 gene not only gives the patient a resistance to HIV but a higher susceptibility to the West Nile virus and editing the beta-globin gene in a patient with sickle cell disease could cure them of its illness but the patient would lose its protection against malaria. Editing any gene will always result in a risk of unforeseen effects. But just because we do not know its side effects doesn't mean we have to renounce germline editing, as George Church said: “The notion that we need complete knowledge of the whole human genome to conduct clinical trials of heritable gene editing seems at odds with medical reality”. Prior to this treatment, countless others have been used without their entire comprehension, so there is no reason for holding CRISPR to a higher standard of safety. As long as we edit the gene to its “normal” version the benefits will outweigh the risks, without taking into consideration human enhancement.

The biggest ethical concern centers around changing the genome of human embryos, due to the fact that any change would be then passed on to succeeding generations. Some people have moral and religious beliefs against using human embryos for research, they may regard the embryo as a person from conception or think of it as something unnatural. But when considering medicine as a whole “the distinction between natural and unnatural is a false dichotomy, and if it prevents us from alleviating human suffering, it’s also a dangerous one.” (128) Some bioethicists regard germline editing as a way to alter humanity itself and its invaluable evolutionary inheritance. While others, including bioethical and research groups, find it essential to solving many questions about human development.

Some bioethicists like Julian Savalescu argue that gene editing “could virtually eradicate genetic birth defects” and that “research into gene-editing is not an option, it is a moral necessity.” However other bioethicists approve of genome editing on embryos for research but are opposed to its clinical application.

This debate was already present in our society since we developed other methods of genetic manipulation, such as TALENs, and we started using preimplantation genetic diagnosis (PGD) and in-vitro fertilization (IVF). This ongoing debate regained attention after the discovery of CRISPR because it makes genetic manipulation an easier and more accurate process. Some scientists claim that genome editing may never have a greater benefit than methods such as PGD and IVF. However, in some cases in which PGD is ineffective genome editing may prove useful. For example, when both parents are homozygous for a disease, so if they were to have children they would all have the disease or cases of polygenic diseases where there is more than one gene causing the disease. On one hand, some ethicists argue that using this technology will start a slippery slope that will have humans using it to upgrade the human genome in a quest for perfection. On the other hand, some believe once it is studied and safe it could potentially eradicate disease and avoid serious genetic defects, while concerns about the technology being abused should be considered through laws and policies (129).

Furthermore, there are concerns about the use of genome editing for reproductive purposes being regulated differently throughout the world, just like PGD and IVF have differences in their policies worldwide. In M. Araki and T. Ishii’s study published in 2014, 39 countries policies regarding the editing of the human genome were studied.

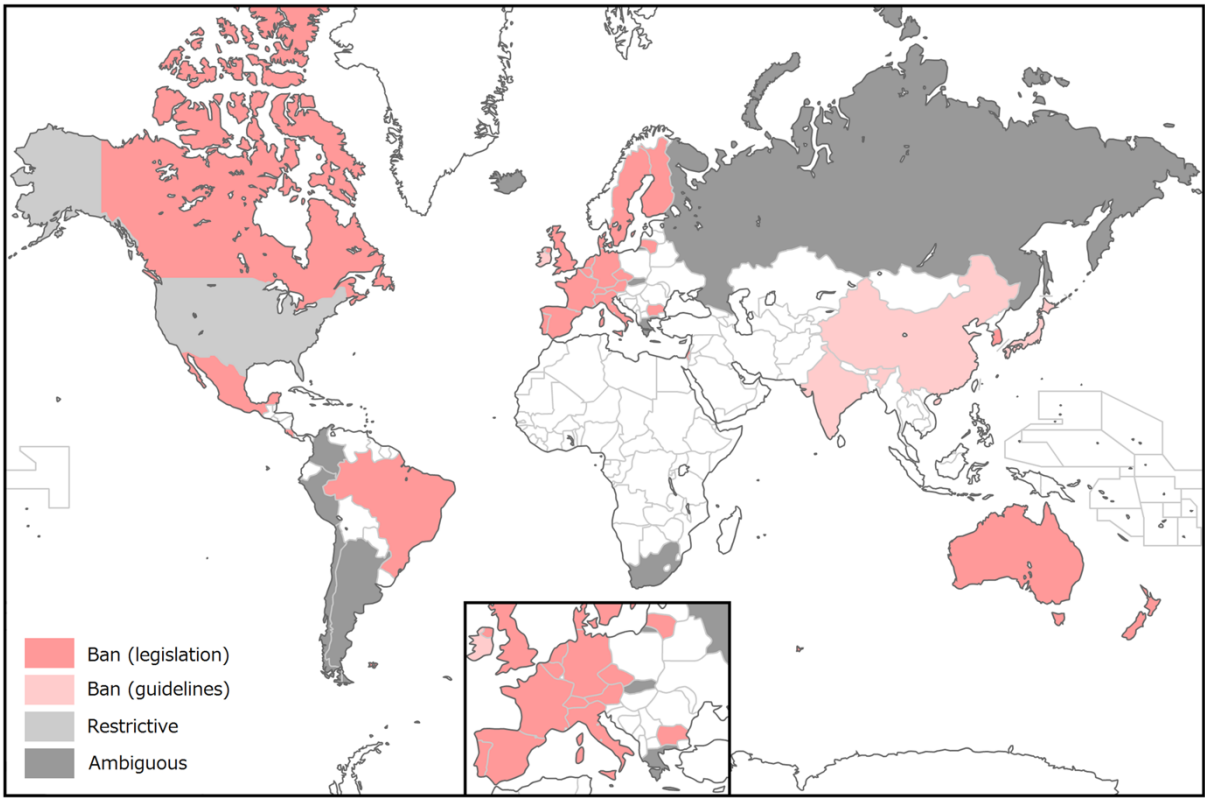


Fig. 14: *International regulation of human genome editing.* From (130).

Out of these 39 countries, 29 had a ban on this kind of research, shown in dark and light pink on the map. But while the 25 countries in dark pink have laws banning this practice the remaining 4 in light pink only have guidelines that ban this kind of studies, there are not enforceable laws. The remaining countries (grey on the map) had ambiguous guidelines pertaining to this studies, although USA (light grey) does have a restrictive set of rules.

This study shows that while there are countries such as Austria where “any intervention involving the human germline is prohibited” there are others such as China, Japan or USA that only have certain guidelines and might use these techniques once they become safer (130). This disparities between countries could encourage CRISPR tourism, patients with means could travel to countries where regulations are more lax or absent altogether.

Due to this disparity in regulations, there are some international organizations, which include the USA, UK, and China, trying to unify the regulation for the application of the CRISPR technologies. This endeavor started in the “International Summit on Human Gene Editing” in December 2015 (127). According to Doudna: “Nations need to maintain regulatory environments that are hospitable enough to permit research and clinical applications but strict enough to prevent the worst excesses.” It is unrealistic to think we will come to an agreement worldwide on how to use this technology but it would certainly be beneficial for everyone if governments tried to unify their policies on germline editing.

Once this technology is safe enough for clinical applications to prevent genetic diseases there should be further discussions and ethical considerations to avoid its abuse. The Hinxton Group, an international consortium on stem cells ethics and law, acknowledge that “when all safety, efficacy and governance needs are met, there may be morally acceptable uses of this technology in human reproduction, though further substantial discussion and debate will be required.” Some uses of this technology in embryos may be accepted, when it prevents a child from inheriting a disease, while other uses might be forbidden, such as genetic enhancement.

Once this technology is fully developed and functional we have to acknowledge it might be used for genetic enhancements. There is not a clear line between what is classified as a medical treatment and a genetic enhancement. If the gene *PCSK9* is edited the patient will reduce their risk of heart disease due to low cholesterol, editing the *APOE* gene will diminish the patients’ risk of Alzheimer’s disease, etc. There are numerous examples were editing a gene reduces the persons’ risk of developing a disease but it also provides those individuals with an above average genome. This is without taking into consideration non-medical enhancements were some scary possibilities will be viable and at everyone’s disposal, like creating super soldiers or the perfect race.

Just like its hard to know where the line between medical treatment and enhancement is, its hard to see how this could be done equitably. There are concerns about this technology only being accessible to the wealthy which would generate even bigger discrepancies in health care between the rich and the poor. Taken to the extreme, editing genomes could create different classes of humans depending on the quality of their engineered genome. The wealthy would live healthier and longer lives.

Moreover, who decides which therapies should we develop? There are obvious candidates like cancer, Huntington or cystic fibrosis but what about familial short stature or hereditary deafness? Many people with this conditions think there is nothing to “correct” about them (128). Should we consider cosmetic changes? Humans tend to try to get physical perfection, assuming this could be obtained with CRISPR-Cas9, should this be pursued?

This technology raises many ethical issues that are non-human related too, like the risks taken by generating enhanced animals or plants or the risk of generating a worldwide ecological impact. Given the great opposition against GMOs in agriculture, this technique could face the same opposition if the general public were misinformed.

Since it is society as a whole who should decide how to use this technology it is up to scientists to educate the people so they can make an informed decision. (128)

CONCLUSION

The CRISPR-Cas arose from studying the archaea and bacteria immune systems and has become the most precise genome editing technology. Studies using CRISPR-Cas technology have increased in recent years, revealing its potential in modern medicine. Many clinical trials are in progress to use ex-vivo and in-vivo somatic cell editing in patients, since this technology has enabled in-depth studies of our genome and the means to edit any specific gene.

In the near future, editing the human genome with CRISPR-Cas9 will aid in the treatment of genetic and infectious diseases, as well as cancer. Although some challenges remain, CRISPR-Cas technology will become a safe and applicable method used in a variety of therapeutic approaches.

The BRCA1 study shown in this report demonstrates the simplicity and potential of this technology, it enables a fast and cheap procedure, were a single nucleotide can be modified, eliminating hereditary breast and ovarian cancer from the patients cells.

The uses of CRISPR-Cas system goes beyond medical applications, contributing in advancing modern agriculture and biotechnology. Some of the numerous applications in this field are crop editing, gene drives or synthetic biology.

CRISPR-Cas9 provides endless possibilities to improve the world. This technology could be used to eradicate genetic diseases, to cure cancer or even solve the worlds hunger crisis. The possibilities are countless. However, genetic engineering is a highly controversial societal issue. Unifying governmental policies around the world on how to use this technology safely should be addressed in the near future, focusing on policies concerning germline editing.

Once used to change the human germline, it will alter the human genome in a heritable way. While some scientists find the use of this technology immoral, others like Doudna, believe it is “unethical to not ease human suffering when it is in our hands.” This poses new questions: how can society choose which ways to use this technology and when to restrict or ban its use?

It is impossible to unlearn this knowledge or pretend like it is not here to stay. The important question now is: are humans ready to have the power to control every species’, including our own genetic future?

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